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THE CLASSIFICATION OF THE STREPTOCOCCI BY THEIR ACTION UPON CARBOHYDRATES AND RELATED ORGANIC MEDIA.*†

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When Gordon[1] first suggested the systematic use of a number of carbohydrate media in the classification of streptococci it appeared that there was at last some hope of clearing up the relationships of this puzzling group. He studied the action of streptococci upon 35 different organic compounds and finally selected seven as promising the most significant results. These included two disaccharides, lactose and saccharose; one trisaccharide, raffinose; one polysaccharide, inulin; two glucosides, salicin and coniferin; and one alcohol, mannit. He applied these tests, with the clotting of milk and the reduction of neutral red, to 300 cultures from normal saliva,[2] and to 101 from air.[3] Houston examined in the same way 300 strains of streptococci from human feces,[4] 172 from milk,[5] and 100 from cow dung.[6] Andrewes and Horder[7] made a comparative statistical study of most of these records and of some obtained by themselves in the examination of over 200 pathogenic forms, the entire series including 1,200 strains. They were led to the conclusion that virulence tests had little systematic value on account of the readiness with which this property may be lost or gained. The fermentation reactions, on the other hand, showed themselves remarkably constant, and the difference between long chained and short chained forms appeared on the whole to be of value. When individual strains were considered an almost infinite series of

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[1] *Rep. of Med. Off. to Local Gov't Bd., Great Britain*, 1902–3, 32, p. 421; *ibid.*, 1903–4, 33, p. 388.

[2] *Loc. cit.*

[3] *Report on an Investigation of the Ventilation of the Debating Chamber of the House of Commons*, 1906.

[4] *Rep. of Med. Off. to Local Gov't Bd., Great Britain*, 1903–4, 33, p. 472.

[5] *Report to the London County Council on the Bacteriological Examination of Milk*, July 11, 1905.

[6] *Rep. of Med. Off. to Local Gov't Bd., Great Britain*, 1904–5, 34, p. 326.

[7] *Lancet*, 1906, 2, p. 708.

varieties appeared; but when the results were analyzed from the statistical standpoint it was possible to distinguish certain types which were obviously important on account of their numerical frequency. To seven of these types they gave names and characterized them as indicated in Table 1.

TABLE 1.

ANDREWES AND HORDER'S TYPES OF STREPTOCOCCI.

Name	Milk Clot	Neutral Red	Saccharose	Lactose	Raffinose	Inulin	Salicin	Coniferin	Mannit	Growth on Gelatin at 20° C.	Long Chains	Pathogenesis for Mouse
Str. equinus..........	..	..	+	..	..	..	+	+	..	..	..	..
" mitis..........	..	..	+	+	..	..	±	..	..	+	..	..
" pyogenes..........	..	..	+	+	..	..	±	..	..	+	+	+
" salivarius..........	+	±	+	+	±	..	..	..	..	±	..	..
" anginosus..........	+	±	+	+	..	..	..	..	..	±	+	+
" fecalis..........	+	+	+	+	..	..	+	+	+	+	..	..
Pneumococcus..........	±	..	+	+	+	±	..	..	..	..	..	+

Since the publication of Andrewes and Horder's paper, Houston has reported 100 more strains from human feces and 71 from water,[1] and Gordon has reported 155 strains from scarlatinal throats.[2] In the United States, Winslow and Palmer[3] have studied 300 strains from human, bovine, and equine feces, using only four fermentable media, while more recently Miss Broadhurst[4] has reported tests on 100 milk streptococci and Hilliard[5] on 65 strains from normal and diseased throats. The results of these observers in some respects conflict with the English work, and even in England Andrewes and Horder's types, after five years, have obtained no general acceptance. The time would seem to be ripe for fresh study of the problem of classifying the streptococci and of the value of Gordon's tests in bringing about its solution.

Two things appear to be necessary if this work is to be carried forward successfully—a uniform and reliable method of determining fermenting power, and a clue to the interpretation of the results obtained. As long as each organic medium is treated as an independent unit, unrelated to any other substance, there can result only a confusing multiplicity of types. It has seemed worth while

[1] *Fifth Report on Research Work, Metropolitan Water Board,* London, 1910.

[2] *Rep. of Med. Off. to Local Gov't Bd., Great Britain,* 1910–11, 40, p. 302.

[3] *Jour. Infect. Dis.,* 1910, 7, p. 1.

[4] *Ibid.,* 1912, 10, p. 272.

[5] *Science,* N.S., 1912, 35, p. 223.

to make a comparative study of the results of the many English and American workers who have dealt with this subject in the hope of attaining these two ends—the recognition of a sound method and the suggestion of a rational basis of relationship between the action of streptococci upon different organic media.

The English results have, without exception, so far as I am aware, been obtained by merely recording positive or negative results as indicated by the reaction of litmus. Gordon[1] describes his method as follows: "Faintly alkalin sugar-free broth was tinted with litmus, charged with the organic substance to be tested, and after inoculation with a given streptococcus, was incubated aerobically for three days at 37°." "Alkalin" presumably means alkalin to litmus since the use of exact titration methods with phenolphthalein as an indicator, almost universal in this country, is rare in England. Thus, when Gordon and Houston report that a streptococcus gives positive results, they mean simply that it changes a medium faintly alkalin to litmus so as to produce reddening.

In the work of Palmer and the writer and that of Miss Broadhurst and of Hilliard, tests for acid production were made by quantitative methods, duplicate tubes being titrated, with phenolphthalein as an indicator. Uninoculated controls were titrated at the same time, and the value obtained subtracted from that found in the inoculated tubes, to give the exact amount produced by the bacteria. When the results of such a study are plotted, they usually show two distinct maxima, one in the neighborhood of the point of no acid formation, the other at a somewhat high acidity. For example, the results obtained by Palmer and the writer for fecal streptococci were as shown in Table 2.

TABLE 2.

ACID PRODUCTION OF FECAL STREPTOCOCCI IN LACTOSE BROTH.

Acid production Percentage normal	−.5 0.0	0.1 0.5	0.6 1.0	1.1 1.5	1.6 2.0	2.1 2.5	2.6 3.0	3.1 3.5	3.6 +
Number of strains	113	64	7	7	26	19	45	20	1

There is clearly one group centering about the point of no acid production but extending to 0.5 per cent normal on each side of it, and another group producing 1.5 to 3.5 per cent normal acid, while few strains fall in the region between. According to well known biometrical principles the division between fermenting and non-fermenting strains should be made in this gap and not at any arbitrary point fixed by an indicator. A study of the data published by Palmer and the writer and of the results obtained by Miss Broadhurst and Hilliard, not yet published in full but kindly placed at the writer's disposal, shows that this division line falls for all carbohydrates studied between 0.5 and 2.0 per cent normal and may be placed without serious error at 1.2 per cent normal for all of them.

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[1] *Rep. of Med. Off. to Local Gov't Bd., Great Britain,* 1903–4, 33, p. 388.

Litmus is much less sensitive than phenolphthalein, and in ordinary sterile nutrient broth it takes about 0.8 per cent normal hydrochloric acid to change a distinctly blue solution to one that is distinctly red. If this relation were general the litmus test ought to give somewhere near the same result as the titration test (using 1.2 per cent normal increase in acidity as positive), and this is why the two methods roughly correspond. The difference between alkalin and acid litmus broth will be more or less sharp, however, according to the judgment of the observer, and the litmus method must be less accurate than the titration method. Furthermore, the presence of some constant difference seems to be indicated by the comparison of English and American results, tabulated in Table 3.

TABLE 3.

COMPARISON OF RESULTS OBTAINED BY ENGLISH AND AMERICAN OBSERVERS.
Percentage of Fermenting Strains.

SOURCE OF STREPTOCOCCI	THROAT			HUMAN FECES		BOVINE FECES		MILK	
Observer	Hilliard	Gordon		Winslow Palmer	Houston	Winslow Palmer	Houston	Broadhurst	Houston
		A	B						
Lactose	63	84	97	62	100	52	100	87	100
Saccharose	38	100	100	..	85	..	94	69	99
Salicin	..	40	62	..	93	..	95	75	72
Inulin	..	13	5	..	..	..	..	33	..
Mannit	0	0	2	28	31	6	0	31	15
Raffinose	4	48	28	6	26	28	80	11	62

A = Normal throat. B = Scarlet fever throat.

With three exceptions, the English results are higher, usually very much higher, and it seems reasonable to attribute this to the difference in technic rather than to any national peculiarities in the streptococci tested. Of course the choice of the titration method (aside from its obviously greater exactness) rests on the assumption that the biometrical method of classification is a valid one and that the dividing line between fermenting and non-fermenting strains should be made at the intermodal point of the frequency curve. If this be granted, the American method appears to be accurate; and if it be accurate the English method appears to yield results which are too high. In view of this constant difference and in view of the obvious inexactness of the litmus test it seems that systematic studies of the fermentative relations of the streptococci should be carried out by titration with phenolphthalein as an indicator. Media may be made up from meat extract (each batch checked by controls inoculated with *B. coli*) and adjusted to an initial reaction between neutral and 0.5 per cent normal acid. Dextrose, lactose, saccharose, salicin, inulin, mannit, and raffinose should all be used for diagnosis, for the present at least, and titration may be made after cultivation for three days at 37° C. When studied in this way, cultures producing over 1.2 per cent normal acidity more than that initially recorded may be considered positive.

The second aim of the present study was to examine the relations between the fermentability of the different organic media. For this purpose, all available results obtained by the titration method have been compared. These include 202 strains of streptococci from human and bovine feces studied by Palmer and the writer, 101 strains from milk studied by Miss Broadhurst, 65 strains from normal and diseased throats studied by Hilliard, and 17 from various sources studied by T. D. Ballinger at the American Museum under the general direction of the writer, making 385 in all. The detailed results of the last two observers, not yet published in full, have been courteously furnished for the purpose of this analysis.

The relative frequency with which the various media are attacked is indicated for the entire series in Table 4.

It appears at once from this table that dextrose, lactose, salicin, and saccharose are readily fermentable, half or three-quarters of all strains giving positive results, while mannit, inulin, and raffinose are much less readily attacked. The data are, however, not wholly comparable. Salicin tests, for example, were made only by Miss Broadhurst and Ballinger working almost wholly with milk strains, which are always high fermenters; hence the salicin percentage is higher than it should be relatively, while one-third of all the strains tested in saccharose were Hilliard's throat strains, which are frequently low fermenters. The only way to get the actual relative availability of the different media is to study the individual correlations; and these have been worked out for all the tests made in Table 5.

TABLE 4.

FERMENTABILITY OF VARIOUS ORGANIC MEDIA BY THE STREPTOCOCCI.

	NUMBER OF STRAINS			PERCENTAGE POSITIVE RESULTS
	Fermenting	Failing to Ferment	Not Tested	
Dextrose	244	53	88	82
Lactose	249	136	. .	65
Salicin	88	30	267	75
Saccharose	101	82	202	55
Inulin	38	80	267	32
Mannit	69	316	. . .	18
Raffinose	46	339	. . .	12

TABLE 5.

CORRELATIONS OF DIFFERENT ORGANIC MEDIA IN REGARD TO FERMENTABILITY.

Number of Strains in Each Class.

		Lactose		Salicin		Saccharose		Mannit		Inulin		Raffinose	
		+	−	+	−	+	−	+	−	+	−	+	−
Dextrose	+	162	82	20	8	42	47	42	202	6	22	25	219
	−	**18**	35	**0**	2	**0**	6	**0**	53	**0**	2	**10**	43
Lactose	+	. .	. .	74	24	92	47	66	183	45	53	50	199
	−	. .	. .	**14**	6	**9**	35	**3**	133	**1**	19	**3**	133
Salicin	+	. .	. .	. .	. .	63	26	41	47	35	53	9	79
	−	. .	. .	. .	. .	**12**	17	**2**	28	**5**	25	**6**	24
Saccharose	+	. .	. .	. .	. .	. .	. .	38	63	37	38	15	86
	−	. .	. .	. .	. .	. .	. .	**5**	77	**1**	42	**1**	81
Mannit	+	. .	. .	. .	. .	. .	. .	. .	. .	20	23	9	60
	−	. .	. .	. .	. .	. .	. .	. .	. .	**18**	57	**37**	279
Inulin	+	. .	. .	. .	. .	. .	. .	. .	. .	. .	. .	9	29
	−	. .	. .	. .	. .	. .	. .	. .	. .	. .	. .	**6**	74

The substances studied are here arranged in what appears to be on the whole their order of relative availability. If any member of the series is fermented, the chances are that those ahead of it will be fermented also. If any member is not attacked, the chances are that those behind it will not be attacked either. For example, a positive result with dextrose does not imply a positive result with any other substance, but a negative result with dextrose does imply a probably negative result with all other substances. Thus of the strains failing to act on dextrose, two-thirds did not ferment lactose, four-fifths did not ferment raffinose, and none attacked any other of the substances tested. Of the strains fermenting inulin, all attacked dextrose, all but one lactose and saccharose, and 35 out of 40 attacked salicin, though only half fermented mannit. There are exceptions, of course, as would be expected, since the variability of organisms under the conditions of such experiments are always considerable. The exceptions to the general rule are indicated by the figures in the lower left hand corner of each correlation table in heavy type. In the cases of lactose and salicin, salicin and saccharose, mannit and inulin, and mannit and raffinose, these exceptions are considerable, and all these are examples of substances lying close to each other in the series. The general trend of the figures may perhaps be brought out by adding together the heavy type figures (giving a total of all cases in which a substance was attacked while one higher in the series was not) and comparing with the corresponding total for the upper right hand figure in each correlation table (total of all cases in which a substance was attacked while one lower in the series was not). The total of the lower left hand figures is 151; of the upper right hand figures 1,590. The relations are perhaps better shown in Table 6.

Thus if any given substance is fermented, only between 23 and 42 per cent of the substances lower in the scale are acted upon, while between 85 and 95 per cent of those higher in the scale are fermented (excepting only in the case of raffinose, which is somewhat aberrant). If a substance is not fermented, only from 20 to 49 per cent of those preceding it in the scale are not acted on, while 80 to 97 per cent of those following it fail to show a reaction.

I have tried to derive a mathematical expression for these relations but so far without success. It seems clear, however, that the substances tested do stand to each other in a definite order of availability. Howe,[1] has described the same phenomenon in the fermentative power of the colon bacillus and its allies, and suggested for it the excellent name of "metabolic gradient."

TABLE 6.

RELATION BETWEEN FERMENTABILITY OF DIFFERENT ORGANIC MEDIA.

	GIVEN SUBSTANCE +		GIVEN SUBSTANCE −	
	Percentage of		Percentage of	
	Subsequent Substances +	Preceding Substances +	Subsequent Substances −	Preceding Substances −
Dextrose	34	..	83	..
Lactose	39	81	92	30
Salicin	42	87	80	20
Saccharose	32	90	97	33
Mannit	26	95	86	37
Inulin	23	85	92	43
Raffinose	..	65	..	49

The order of availability, so far as the streptococci are concerned, corresponds closely to what might be expected from the chemical composition of the substances concerned. First comes the monosaccharide, dextrose, then the disaccharides, lactose and saccharose, and the glucoside, salicin. The latter it will be remembered is an ethereal derivative ($C_{13}H_{18}O_7$) which is easily broken up by acids, alkalies, or enzymes to yield a monosaccharide. Considerably lower in the scale stand the hexatomic alcohol mannit ($C_6H_{14}O_6$) and the starch-like body, inulin. Least available of all is the trisaccharide, raffinose, but this body does not fit into the metabolic gradient as closely as the other substances. In Table 5 it appears that a considerable proportion of strains which ferment raffinose are negative in dextrose, salicin, mannit, and inulin.

It is interesting to note that the order of availability of the various substances is not the same for the colon bacilli and the streptococci. Dextrose is most generally attacked in each case and lactose comes next. Among the streptococci, saccharose is almost as easily attacked as lactose, while raffinose is rarely fermented. That is, the size of the molecule appears to be the main factor involved. With colon bacilli, on the other hand, the lactose fermenters are divided into two groups of about equal size by the saccharose test; and it has been shown by Walker and the writer,[1] and others that when saccharose is attacked raffinose is usually fermented also. With these bacilli it is not the size of the molecule which is significant but its configuration, the sugars with an aldehyde grouping being easily attacked, while the ketonic sugars (saccharose and raffinose) are less commonly fermented, whether they are disaccharides or trisaccharides.

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[1] *Science*, N.S., 1912, 35, p. 225.

It seems possible that the conception of the metabolic gradient may materially simplify the classification of bacteria by their fermentative reactions. For the present as large a series of substances as possible should be tested in order to confirm the conclusions here suggested. Ultimately, however, it may be possible, for example, to recognize a group of streptococci fermenting monosaccharides only, and another group attacking disaccharides also (using as a test one disaccharide only and leaving out perhaps saccharose and salicin), while a third group ferments the trisaccharides also. With the colon group, on the other hand, saccharose is of prime importance as an index of the power to attack ketonic sugars, while additional tests with raffinose add little to our knowledge.

The results already attained by biometric studies, using the quantitative titration method, seem sufficiently promising to warrant carrying them further. Thus the work of Palmer and the writer suggested that mannit-fermenting streptococci are particularly characteristic of human feces and raffinose-fermenters of the feces of cattle. Hilliard finds that throat streptococci do not usually attack any substance more complex than the disaccharides and that milk streptococci can be generally distinguished from throat strains by the amount of acid formed at 20°. Broadhurst has shown that although milk streptococci are more closely related to human than to bovine strains, they have an unusually wide range of fermentative power and produce a very high actual acidity. All these more or less preliminary results suggest that the biometric study of fermentative powers may not only throw light on the systematic relationship of the cocci but may yield results of practical sanitary importance.

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[1] *Science*, N.S., 1907, 26, p. 797.